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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/02, 37/22, 9/52, 31/70, 35/14, 39/00, 39/395, 48/00, C07K 7/00, 13/00, 15/28, C07H 17/00, 21/04

A1

(11) International Publication Number: **WO 94/23738**

(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/US94/04239

(22) International Filing Date: 19 April 1994 (19.04.94)

(30) Priority Data:
08/047,536 19 April 1993 (19.04.93) US

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(81) Designated States: AU, CA, JP, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: ENCAPSULATION OF NUCLEIC ACIDS WITH CONJUGATES THAT FACILITATE AND TARGET CELLULAR UPTAKE AND GENE EXPRESSION

(57) Abstract

This invention is a method for encapsulating nucleic acids linked to or co-existing with other molecules that facilitate the uptake and integration of genetic material into living cells by means of slow-release of DNA/RNA or oligonucleotides combined with cell surface ligands/proteins/transcription factors and antibodies. Compositions for use with this method are disclosed.

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TITLE

ENCAPSULATION OF NUCLEIC ACIDS
WITH CONJUGATES THAT FACILITATE AND
TARGET CELLULAR UPTAKE AND GENE EXPRESSION

FIELD OF THE INVENTION

The present invention relates to the controlled release of nucleic acids (DNA, RNA, synthetic oligonucleotides or derivatives thereof) conjugated or in combination with proteins, antibodies, or other molecules within slow release biodegradable polymeric microparticles. The present invention also relates to the use of encapsulated genes for genetic transformation.

BACKGROUND OF THE INVENTION

One of the rate-limiting steps in biotechnology is the insertion of genetic material into cells and tissues. To date, known methods have not provided satisfactory means to facilitate the controlled uptake and integration of genetic material into living cells of animals and plants for the useful expression of exogenous genes.

Various efforts have been directed at improving the uptake of exogenous nucleic acids into specific cells. Several molecules and receptors have been shown to facilitate nucleic acid uptake into specific cells. For example, asialoglycoprotein increases uptake into liver cells (Wu et al., J. Biol. Chem., 262:4429-4432 (1987)), transferrin-polylysine adenovirus facilitate uptake into epithelial cells (Curiel et al., Proc. Natl. Acad. Sci. USA, 88:8850-8854 (1991)), and viral particles such as herpes virus are transported into neural cells (Geller et al., Science 241:1667-1669 (1988)). Antibodies that bind to specific cellular ligands can also facilitate uptake to specific cells and tissues. In addition, liposomes with receptor ligands have been used to

transfer DNA to cells (Malone et al., Proc. Natl. Acad. Sci. USA, 86:6077-6081 (1989)). These preferential and specific uptake molecules bind to specific cell-surface receptors and are internalized by the receptor mediated-endocytosis pathway.

Various chemical modifications have been performed on nucleic acids which help promote their up-take and subsequent expression by cells. It has been shown (Huckett et al., Biochem. Pharmacol., 40:253-263 (1990)) that when DNA was non-covalently bound to a chemically modified albumin cross-linked to insulin, the trimolecular complex was bound by the insulin receptor on HepG2 cells, taken into the cell by receptor-mediated endocytosis, transported to the nucleus and expressed in the form of mRNA transcription and protein translation. Recently, molecules that target integration into specific sites on the chromosome have been identified. Moreover, DNA-binding proteins and other molecules can promote transcription.

What these methods lack is the means to protect the components from degradation. A solution to this problem would not only protect the components but also permit slow-release of the encapsulated nucleic acids and ligands.

Gene Delivery Methods

Existing technologies for transporting genetic material into living cells involve methods for chemical uptake, retroviral infection, and physical insertion including particle bombardment. Several of these methods can be improved with use of encapsulated nucleic acids with conjugates as proposed herein. Particle-mediated bombardment of DNA to plant, animal and microbial cells as well as to living animals have been reported [Fitzpatrick-McElligott, Bio/Technology 10:1036-1040 (1992), Klein et al, Bio/Technology

10:286-291. (1992), Williams et al. Proc. Natl. Acad. Sci. USA. 88:2726-2730 (1991)].

Currently known methods have the disadvantage of permanent germ line integration of genetic material which in certain cases is not desirable. Once a gene has integrated into the cell germ line, as is the case with retrovirus-mediated gene transfer, it is usually there until the cell dies. Patients not requiring permanent gene replacement cannot be treated by retroviral gene therapy. Another issue is safety. Retroviruses are oncogenic viruses and are inherently dangerous for use in the general human population.

Currently, protein injections are used for the treatment of some diseases. Protein injections can result in an uneven availability, leading to potentially toxic doses immediately after injection, with insufficient amounts later on. A method of somatic transformation with slow-release genetic material to allow sustained production and delivery of proteins and peptides is needed.

Biodegradable Microparticles

The use of biodegradable microparticles containing drugs as a slow release delivery system is known. U.S. Patents Nos. 4,389,330 and 4,542,025 disclose a variety of microparticles, or microcapsules, their preparations and their usage.

European Patent Application 248,531 discloses RNA and/or DNA or antisense RNA in microcapsules for the induction of interferon production, which is said to be a potential inhibitor of viral replication. However, the European patent application fails to provide a method for uptake and delivery to the intracellular cytoplasm of the cells. Therefore, use of the method as outlined by the European patent application would not be

sufficient for the use of genetic material and gene expression.

Encapsulation of genetic material would protect the nucleotides from enzymatic degradation before they are released. Controlled release of genes would also reduce lethality to the organisms by allowing controlled expression of product.

SUMMARY OF THE INVENTION

The present invention is a microparticle composition suitable for the controlled release of a nucleic acid to a target cell, the microparticle comprising a microparticle composition suitable for the controlled release of a nucleic acid to a target cell, the microparticle comprising:

(a) a nucleic acid comprising synthetic or natural DNA or RNA exogenous or native to a target cell, the nucleic acid conjugated by way of chemical bonds with promoting material which promotes the uptake or the transport to the nucleus, or expression of the nucleic acid in the cell, the molecules selected from the group consisting of glycoproteins, lipoproteins, nucleoproteins and peptides, hormones, antibodies, growth factors, nucleic acid binding factors, proteinaceous cellular ligands, glycolipids, peptidoglycans, lectins, fatty acids, phospholipids, glycolipids, triglycerides, steroid hormones, cholesterol, single stranded or double stranded RNA, single stranded or double stranded DNA, and intercalating agents, the nucleic acid present in an amount of about 0.0001 wt % to 50 wt % based on the parts of nucleic acid per weight of an encapsulating polymeric matrix of element (b);

(b) a biocompatible, biodegradable polymeric matrix encapsulating the nucleic acid of element (a), the polymeric matrix selected from the group consisting

of poly-d, L-lactic acid, poly-L-lactic acid, polyglycolic acid, copolymers of mixed d,L-lactic acid and glycolic acid, copolymers of L-lactic acid and glycolic acid, copolyoxalates, polycaprolactone, poly(lactic acid-caprolactone), poly(glycolic acid-caprolactone), casein, albumin, and waxes; and

(c) the microparticle ranging in diameter from 1 to 500 microns. Additionally, the invention encompases a microparticle composition suitable for the controlled release of a nucleic acid to a target cell, the microparticle comprising a microparticle composition suitable for the controlled release of a nucleic acid to a target cell, the microparticle comprising:

(a) a nucleic acid comprising synthetic or natural DNA or RNA exogenous or native to a target cell, the nucleic acid conjugated by way of chemical bonds with promoting material which promotes the uptake or the transport to the nucleus, or expression of the nucleic acid in the cell, the molecules selected from the group consisting of glycoproteins, lipoproteins, nucleoproteins and peptides, hormones, antibodies, growth factors, nucleic acid binding factors, proteinaceous cellular ligands, glycolipids, peptidoglycans, lectins, fatty acids, phospholipids, glycolipids, triglycerides, steroid hormones, cholesterol, single stranded or double stranded RNA, single stranded or double stranded DNA, and intercalating agents, the nucleic acid present in an amount of about 0.0001 wt % to 50 wt % based on the parts of nucleic acid per weight of an encapsulating polymeric matrix of element (b);

(b) a biocompatible, biodegradable polymeric matrix encapsulating the nucleic acid of element (a), the polymeric matrix selected from the group consisting of poly-d, L-lactic acid, poly-L-lactic acid,

polyglycolic acid, copolymers of mixed d,L-lactic acid and glycolic acid, copolymers of L-lactic acid and glycolic acid, copolyoxalates, polycaprolactone, poly(lactic acid-caprolactone), poly(glycolic acid-caprolactone), casein, albumin, and waxes; and

(c) the microparticle ranging in diameter from 1 to 500 microns. In the first case, the promoting material is directly conjugated to the nucleic acid. In the second case, the promoting material co-exists with the nucleic acid within the polymeric matrix. Both of the microparticle compositions described above may further comprise an inert particle as the core of the microparticle, the inert particle made of tungsten, gold, platinum, ferrite, polystyrene, or latex.

The invention includes a method for preparing nucleic acids in a size effective for cellular or tissue insertion to effect gene expression in plants and animals comprising forming a controlled release, biocompatible biodegradable microparticle comprising one of the two microparticle compositions set out above.

The method described above may further include coating the polymeric matrix containing the nucleic acids with the promoting material to promote the uptake of the nucleic acid into the cell or the transport of the nucleic acid to the nucleus.

The invention also includes a method for the controlled delivery of an exogenous or native gene into the cells of plants or animals to effect gene expression, the method comprising:

(a) encapsulating a genetic construct exogenous or native to a target cell within a biocompatible, biodegradable polymer matrix, the encapsulating step forming a microparticle composition;

(b) containing the microparticle composition of step (a) in a dosing device suitable for delivery of the microparticle to a target cell;

(c) discharging the microparticle composition of step (a) into a plant or animal from which site the genetic construct contained within the microparticle composition can interact with the nucleus of a target cell upon degradation of the polymer matrix.

The invention uses methods of direct delivery of microparticles either by injection, particle bombardment or other methods to cells and tissues either in culture or in the living animal or plant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the microcapsules with the genetic material stained with DAPI (4',6-diamididino-2-phenylindole) and tungsten microparticle of >1 μm in size. Black arrows indicate the blue DAPI stained genetic material. The dark tungsten core is indicated by white arrows.

Figure 2 is a scanning electron micrograph showing the size distribution and surface qualities of the microencapsulated particles. The microparticles contain tungsten and plasmid DNA and herring sperm DNA.

Figure 3 is an agarose gel showing the amplified gene after release into the solution at the specified times sampled. Lane 1 show size markers, lane 2 contain no DNA. Lanes 3-7 show the amplified gene released into the solution after 1, 3, 4, 5.5, and 7 hours. Lane 8 shows measurable release after 72 hours. Lane 9 indicates the amplified ß-galactosidase coding region from the plasmid DNA.

Figure 4 shows the expression of the ß-glucuronidase gene in plant cells. Four days after bombardment of cauliflower with microencapsulated genetic material the cauliflower tissue is placed in a reaction buffer

containing X-glucuronic acid (the enzyme substrate). Arrows show the expected blue staining demonstrating gene expression in plant cells with microencapsulated genes.

Figure 5 shows stable clone of transformed animal cells, indicated here with a white arrow. Chinese hamster ovary cells were bombarded with a gene construct containing ß-galactosidase and a neomycin resistance coding sequences. These cells, after bombardment, were selected for neomycin resistance for four weeks. Surviving cells demonstrate neomycin resistance, i.e., the activity of introduced gene. These cells multiply to form a clone, each cell carrying the transgene. All cells of the clone also show ß-galactosidase activity another gene construct in the introduced plasmid DNA. Several clones were observed after four weeks in selection media.

Figure 6 is an agarose gel showing the amplified gene in three clones (Lanes 3-9) after four weeks selection in neomycin media. Lane 1 shows the molecular weight size markers. Lane 2 shows the amplified ß-galactosidase coding region from the plasmid DNA, a positive control marker. Lanes 3-9 show amplified DNA from transformed clones. The amplification of the DNA was done using the polymerase chain reaction. The primers were designed to amplify a sequence in the ß-galactosidase coding region. Lane 10 contains DNA from CHO cells which were not bombarded with exogenous DNA. Lane 11 is a negative control without ß-gal DNA.

DETAILED DESCRIPTION OF THE INVENTION

The proposed invention is a safe and effective method for the controlled delivery of genetic information to the intracellular cytoplasm of humans and other animals for a finite period of time. In the method of the present invention, nucleic acids in

combination with cellular ligands are delivered to the cells of animals and plants by known means, encapsulated in a non-toxic, biodegradable polymer matrix. The method solves a persistent problem in genetic engineering by permitting controlled uptake and prolonged gene expression in the cells and tissues of animals, microbes and plants. Additionally, the invention protects the encapsulated nucleic acids and ligands from enzymatic degradation. Controlled release of genetic material will reduce lethality to the organism by controlling expression of the gene product. Used with known technologies for transporting genetic material into living cells, the invention extends their effectiveness. Very importantly, the invention provides the significant utility of enabling the genetic transformation of somatic cells without germ line integration. Somatic transformation with controlled-release genetic material will allow sustained production and delivery of proteins and peptides to cells.

The present invention uses encapsulated genetic material consisting of a promotor and/or regulatory region and coding region of specific nucleotide sequence for the purposes of obtaining gene expression products, transgenic organisms and for gene therapy.

The following definitions of biological and genetic terms will be useful in understanding this invention:

The term "administered" means any method of delivering the nucleic acid-containing microparticles of the invention to an animal, such as, for example, parenteral (intravenous, intramuscular, or subcutaneous) administration or by the particle delivery method.

The term "animal" as used herein in its usual biological annotation, and encompasses all species of animals large enough to be treated, particularly human,

food animals, and mammals in general; birds, pets, fish and the like.

The term "biocompatible" may be defined as non-toxic to the human body, non-carcinogenic and non-inflammatory in body tissues.

The term "biodegradable" means that the polymeric material degrades by bodily processes to products readily disposable by the body, and do not accumulate excessively in the body. The biodegraded products also should be biocompatible with the body in the sense that the polymeric matrix is compatible with the body.

The term "control" and "inhibit" as used herein as applied to an illness mean the prevention, curing, arrest, or other beneficial pharmacological effect on the illness.

The term "controlled-release" as used herein with respect to microcapsules of the present invention means that the nucleic acid active ingredient is released from the microcapsule polymeric matrix over an extended period of time so as to give continuing or delayed dosage to the treated subject. The controlled-release period can be from 1 to 500 days and preferably is from 3 to 60 days.

"DNA sequence" is a linear sequence comprised of any combination of the four DNA monomers, i.e., nucleotides of adenine, quanine, cytosine and thymine, which codes for genetic information, such as a code for an amino acid, a promoter, a control element (enhancer, nuclear recognition element, etc.) or other gene product. A specific DNA sequence is one which has a known specific function, e.g., codes for a particular polypeptide, a particular genetic trait or affects the expression of a particular phenotype.

"Exogenous genetic material" is genetic material not obtained from or does not naturally form a part of

the specific germ cells or gametes which form the particular zygote which is being genetically transformed.

"Gene" is the smallest, independently functional unit of genetic material which codes for a protein product or controls or affects transcription and comprises at least one DNA sequence.

"Dosing device" is a vehicle for introducing or administering the microparticle to an animal or plant cell.

"Genetic material" is a material containing any DNA sequence or sequences either purified or in a native state such as a fragment of a chromosome or a whole chromosome, either naturally occurring or synthetically or partially synthetically prepared DNA sequences, DNA sequences which constitute a gene or genes and gene chimeras, e.g., created by ligation of different DNA sequences.

"Phenotypic expression" is the expression of the code of a DNA sequence or sequences which results in the production of a product, e.g., a polypeptide or protein, or alters the expression of the zygote's or the organism's natural phenotype.

The term "food animal" means any animal that is consumed as a source of protein in the diet of humans or other animals. Typical food animals include bovine animals, for example cattle; ovine animals, for example sheep; porcine, for example pigs; fowl, for example chickens and turkeys; rabbit and the like.

"Illness" as used herein means a malady to the species caused by internally or externally originating entities. An illness may be localized, such as some tumors, or it may be wide-spread throughout the animal body as in many viral diseases. Illnesses of particular significance to the present invention are cancer and

virally caused illnesses. The term "locale of the illness" refers to a localized illness and means the zone location of the illness.

The terms "microcapsule" or "microparticle" as used herein mean either solid or of the reservoir type particles which contain an active agent, herein genetic material, either in solution or in crystalline form. The active agent is dispersed either or dissolved within the polymer which serves as the matrix of the particle, or is contained within the polymer in reservoir fashion with polymer serving as the outer wall.

COMPOSITION-GENETIC MATERIAL

The invention is directed toward the controlled-release of exogenous, often chimeric, genetic constructs into animal and plant cells. Such gene constructs would normally include a coding region for transcription of the protein or product, together with regulatory sequences. Regulatory regions may be promoter sequences sufficient to initiate transcription and terminator sequences that indicate the end of the product. The nucleic acids useful in the process and compositions of the present invention are, in general, recombinant or synthetic molecules. The present invention does not use the mechanism of interferon induction disclosed in EPA No. 248,531 to inhibit cancer and viral illness.

COMPOSITION-CONJUGATES

Promoting materials promote the uptake or transport to the nucleus, or expression of the nucleic acid in the cell. These molecules are selected from the group consisting of glycoproteins, lipoproteins, nucleoproteins and peptides, hormones, antibodies, growth factors, nucleic acid binding factors, proteinaceous cellular ligands, glycolipids, peptidoglycans, lectins, fatty acids, phospholipids, glycolipids, triglycerides, steroid hormones,

cholesterol, single stranded or double stranded RNA, single stranded or double stranded DNA, and intercalating agents, the nucleic acid present in an amount of about 0.0001 wt % to 50 wt % based on the parts of nucleic acid per weight of an encapsulating polymeric matrix of element (b).

In a preferred embodiment, nucleic acids containing new genetic information are conjugated to a ligand which binds to a cell-surface receptor with high specificity on the cell-type in which one wishes to express the gene. Ligands can be coupled to DNA by several methods. Gene transfer by means of receptor-mediated endocytosis can be accomplished by forming bifunctional molecular conjugates consisting of a binding ligand for a cell-surface receptor that is covalently linked to a DNA binding moiety. Such ligands can be targeted to specific cells. For example, to target hepatic cells, a galactose-terminal (asialo-)glycoprotein, the asialoorosomucoid ligand is covalently linked to poly-L-lysine. The conjugate is then complexed in a 2:1 molar ratio to the plasmid. Asialoglycoprotein is recognized by cell-surface asialoglycoprotein receptors unique to the hepatic cell type (see Wu et al., J. Biol. Chem., 262:16985-16987 (1988)). When the cell-surface receptor recognizes the ligand, the exogenous DNA that is complexed to the DNA binding domain is co-transported into the cells.

Another method for DNA conjugation uses polycation-transferrin conjugates in a complex with DNA to introduce genes into cells. In a typical complex formation reaction 10 µg of transferrin-polylysine or transferrin-protamine conjugate in 250 µL of H_2O is added to 3 µg of plasmid DNA contained in 250 µL of 0.3 M NaCl (while agitating). After 30 min at room temperature the complex is formed and can be

microencapsulated. By coupling the natural iron-delivery protein transferrin to the DNA-binding polycations, polylysine or protamine, a protein conjugate is created that binds nucleic acids and carries them by endocytosis into the cell during the normal transferrin cycle (Wagner et al., Proc. Natl. Acad. Sci. USA., 87:3410-3414 (1990)). Without microencapsulation, endocytosis into the lysosomes, lysosomal enzymes, proteases and nucleases hydrolyze and destroy the DNA.

The method of the present invention protects the DNA and conjugate by encapsulating the complex in a biodegradable polymeric matrix. In addition, co-encapsulation of the complex with chloroquine or adenovirus facilitates breakdown of the lysosomes and allows release of the intact DNA into the intracellular cytoplasmic compartment and access to the nucleus.

Non-covalent complexing of DNA to ligand is achieved by modifying the protein with a water-soluble carbodiimide, N-ethyl-N'; (3-dimethylaminopropyl) carbodiimide hydrochloride (CDI) under conditions which allow the formation of basic N-acylurea moieties. The resulting N-acylurea protein interacts to form salt bridges with the phosphodiester backbone of the DNA [see Huckett et al., Biochemical Pharmacology, 40:253-263 (1990)]. Another method involves derivitization of the nucleic acid 5' terminus to form a hydrazide intermediate which can be coupled with aldehyde-modified proteins (see Ghosh et al., Anal. Biochem., 178:43-51 (1989)).

Various chemical modifications to either 3'- and/or 5'-termini or the individual nucleic acid bases have been performed on DNA. Some of the chemical moieties introduced are fluorescent groups: the acridine derivatives; bathophenanthroline-Ru(II) complexes;

DANSYL, MANSYL, AEDANS-dUTP. These moieties are generally attached through thio- or amino- linkages to terminal hydroxyl or phosphate groups, or to the specific bases. Other molecules which have been attached to DNA include intercalators (e.g., acridine, phenazium); photochemically activated cross-linking (the psoralens) or cleaving (e.g., methylporphyrin XXI) agents; alkylating agents (e.g., chloroalkylaminoaryl); redox active nucleic acid cleaving groups (e.g., 10-Cu(II)-phenanthroline).

DNA end-labelled with biotin can form complexes with antibodies or enzymes through an avidin biotin complex allowing recognition of cell-surface and nuclear membrane transport proteins. Such transport molecules facilitate up-take of the DNA.

Lipophilic moities such as cholesterol, and long-chain alkyl groups are generally attached at the 3' or 5' termini (see Letsinger et al. (1989) PNAS86:6553). Liposomes containing the DNA can be prepared by combining 30 µg DNA and 100 µL of N-[1-(2,3-dioley-oxy)propyl]-N,N,Ntrimethyl-ammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine in serum free media [see Nabel et al., Science, 249:1285-1288 (1990)]. These lipophilic modifications increase the hydrophobicity of the DNA, promoting enhanced uptake through the cell membrane.

The method of this invention will protect the complex and its components and allow prolonged release of the complex by microencapsulation of the DNA conjugates in a biodegradable polymer matrix.

METHOD-MICROENCAPSULATION

By adjusting the composition of the matrix polymer, the rate of release of the drug can be predetermined and targeted for a particular application (see Lewis in "Biodegradable Polymers as Drug Delivery Systems", Eds.

M. Chasin and R. Langer (1990)). The genetic material will be available and released into the animal for an extended length of time as the matrix degrades, and the matrix will protect the unexposed gene from degrading before it has had an effect. Because the polymer used in the method of the invention is biodegradable, all of the entrapped genetic material can be released into the animal. The target cells of humans or animals would internalize the nucleic acid-ligand conjugate by receptor-mediated endocytosis and the nucleic acid would be transported to the nucleus and expressed. The duration of action can be controlled by manipulation of the polymer composition, polymer:drug ratio and microsphere size.

The present invention offers the advantage of durations of action ranging from only 30 to 60 days to more than 250 days depending upon the type of microsphere selected. The delivery system enables introduction of new genetic information into humans or other animals with nucleic acids in a sustained delivery formulation to promote either permanent or transient gene expression and/or to knock out of an existing cellular sequence.

The compositions of the present invention are microparticles (microcapsules), prepared by a conventional technique, having a biocompatible, biodegradable polymeric matrix with nucleic acid distributed within the matrix. The microparticles can be of any conventional type and may include other pharmaceutically effective ingredients, such as an antibiotic, vaccines, and/or other conventional additives. The formulations of the present invention contain nucleic acid dispersed in a microparticle matrix material.

The preferred construction of microcapsules of the invention are described in U.S. Pat. No. 4,389,330, U.S. Pat. No. 4,919,929, and U.S. Pat No. 4,530,840. The microparticles of the invention are composed of a polymer which is, preferably, an aliphatic polyester such as either a homopolymer or copolymer of lactic or glycolic acids. Other degradable polymers may be used, such as, for example, polycaprolactone, polydioxonene, polyorthoesters, polyanhydrides, and natural polymers including albumin, casein, and waxes. The amount of nucleic acids incorporated in the microparticles usually ranges from less than 0.00005 wt% to as high as 75 wt%, preferably 0.0001 nucleic acid to 50 wt% of the polymer. By "weight %" is meant "parts of nucleic acids per parts of polymer by weight". For example, 10 wt% would mean 10 parts nucleic acids per 90 parts polymer by weight.

The polymeric matrix material of the microparticles in the present invention must be biocompatible and biodegradable polymeric material. Suitable examples of polymeric matrix materials include poly-d, L-lactic acid, poly-L-lactic acid, polyglycolic acid, copolymers of mixed d,L-lactic acid and glycolic acid, copolymers of L-lactic acid and glycolic acid, copolyoxalates, polycaprolactone, poly(lactic acid-caprolactone), poly(glycolic acid-caprolactone), casein, albumin, and waxes.

The molecular weight of the polymeric matrix material is of some importance. The molecular weight (MW) should be high enough so that it forms satisfactory polymer coatings, i.e., the polymer MW is proper for the polymer to be a good film former. Usually, a satisfactory molecular weight is greater than 5,000 daltons. The various film-forming polymer compositions have molecular weights readily determined by known techniques. Also, the polymer molecular weight also

plays a significant role (along with composition, purity, optical form, etc.) in rate of degradation. In general, the higher the MW, the slower the degradation. Average molecular weights of about 5,000 to 500,000 are preferred.

Nucleic acids can also be released from the particle by leaching through the polymer matrix, with the nucleic acids being released before the polymer is significantly degraded or simultaneously with polymer degradation. By an appropriate selection of polymeric material, a microparticle formulation can be made such that the resulting microparticles exhibit two phases of release properties. The selection of polymers and manipulation of nucleic acids/polymer ratio are useful in affording multiphasic release patterns.

The microparticle products of the present invention can be prepared by any process capable of producing microparticles in a size range 1 μm to 500 μm acceptable for use in an injectable composition. Generally, microencapsulation processes are classified according to the principal types: (1) phase-separation methods including aqueous and organic phase separation processes, melt dispersion and spray drying; (2) interfacial reactions including interfacial polymerization, *in situ* polymerization and chemical vapor deposition; (3) solvent extraction method; and (4) physical methods, including fluidized-bed spray coating, multi- and single-orifice centrifugal coating, electrostatic coating and physical vapor deposition.

A preferred method preparation is the method described in U.S. Pat. No. 4,919,929. Phase separation methods, as the term implies, rely on differential solubility characteristics that cause a wall- or shell-forming matrix material to separate from solution or suspension and deposit around particles or droplets of

the substance to be encapsulated. The separation, itself, may be brought about physically, as by the addition of a non-solvent or by a change in temperature, or chemically, as by a change in pH.

Organic phase-separation processes (see U.S. Pat. No. 4,919,929) usually employ a dispersion or an emulsion of the nucleic acids in a solution or a high-molecular-weight polymer in an organic solvent. To this mixture is added a non-solvent or liquid polymer that causes the high-molecular-weight polymer to separate from solution and collect as a shell around the suspended therapeutic agent(s). The shell, still swollen with solvent, is then hardened by a further addition of non-solvent or by some other process that strengthens the shell and improves the barrier properties, controlling release by its nucleic acids permeability and/or degradation rate.

Typically in the above described organic phase-separation process, an aqueous solution or suspension of a lipophobic antigen is added to a non-aqueous solution of a suitable matrix polymer, and the mixture is agitated to cause the formation of a water-in-oil emulsion. Depending upon its solubility in water, the agent may be present at a concentration of 0.1 to 50% in the aqueous phase, which may be 0.1 to 20% by weight of the total mixture. The external organic phase may contain 5 to 10% of the matrix polymer. Usually, however, the ratio of agent in the internal phase (aqueous solution or suspension) to polymer is 2:1 to 1:4.

An aqueous phase separation process (see U.S. Pat. No. 4,919,929) employs a dispersion or an emulsion of a water-insoluble therapeutic substance in an aqueous solution or dispersion of a polymer. The polymer is caused to separate as gel particles; these collect

around the therapeutic agent to form a shell; the shell is hardened; and the microparticles are isolated. In the coacervation process, (U.S. Pat. No. 4,919,929) which is the most common of the aqueous phase-separation processes the water-soluble therapeutic agent, which may be in the form of particles or droplets, is usually dispersed in an aqueous sol of a hydrophilic colloid which becomes ionized in water; a second sol of opposite charge is added; and the mixture is caused to gel by a dilution with water, an addition of salt, an adjustment of pH, or a change in temperature, or any combination of these procedures. Appropriate conditions of coacervation are determined readily by routine trial by those of ordinary skill in the art because the various usable polymers differ significantly in physical and chemical properties according to source and method of isolation or preparation. A region of coacervation is determined by combining solutions or sols of two polymers at various concentration, temperatures, and levels of pH, and observing the conditions required for gelation. From these determinations can be drawn a ternary phase diagram, showing the area of compatibility and the region of coacervation, at a given temperature and pH. The changes in concentration, temperature or pH to effect gelation are then apparent.

Each preparation of microparticles requires careful control of conditions, and somewhat different conditions are required for various material being encapsulated. The degree of agitation, for example, affects the size of emulsion droplets. The droplets become smaller in size with increased agitation. The surface properties of the droplets may require alterations in the procedures to insure deposition of matrix material about the droplets and to minimize formation of particles not participating in microencapsulation. The volume of

water added in the dilution step is not critical, but generally larger volumes are required to maintain a stable emulsion when larger droplets are encapsulated.

The above-described phase separation can be adapted to an alternate technique in which the first step of forming a stable emulsion or suspension of an nucleic acids is accomplished by dispersing the nucleic acids in a solution of the matrix material. Thereafter, the emulsion is added drop-wise to a non-solvent with stirring to precipitate the polymer coating material to form microparticles.

Another type of phase separation technique is the melt-dispersion microencapsulation technique (see U.S. Patent No. 4,919,929). A heat-liquefiable, waxy coating material, preferably of a low-melting wax such as glycerol distearate, is suspended in an inert liquid such as a silicone oil or a fluorocarbon in which neither the wax nor the nucleic acids is appreciably soluble. The mixture is heated and stirred vigorously to melt and emulsify the wax. The nucleic acids are powdered and screened to the desired size range, and the waxy coating material is dispersed with high shear agitation. The liquefied wax coats the nucleic acids to form the waxy liquid-coated microparticles. Thereafter, the formed microparticles are solidified by continued agitation which cools the particles. The microparticles are then isolated by filtration and dried as described earlier.

Another method of forming the microcapsules is by interfacial microencapsulation (U.S. Patent No. 4,919,929). This involves bringing two reactants together at a reaction interface where polycondensation of the reactants, usually monomers, occurs to form a thin, insoluble polymeric film. One technique of establishing the interface for the encapsulation process

is the dispersion or emulsification of the nucleic acids with one of the reactants which form the condensation polymer in a continuous phase containing the second reactants.

Still another method of microencapsulation is by solvent extraction (U.S. Patent No.4,919,929). In this method the desired nucleic acids compound is added to the polymer matrix material which has been dissolved in a suitable solvent. The nucleic acids compound may be soluble or insoluble in the solvent for the polymetric material. Optionally, the nucleic acids may be dissolved or dispersed in a second media fluid by adding it to the polymeric matrix solvent.

The mixture of ingredients in the solvent is emulsified in a continuous-phase processing medium, the continuous-phase medium being such that a dispersion of microdroplets containing the indicated ingredients is formed in the continuous-phase medium. The continuous-phase processing medium, commonly water, and the organic solvent must be immiscible. Nonaqueous media, such as xylene and toluene and synthetic oils and natural oils can be used as the continuous phase processing medium. Usually, a surfactant is added to the continuous-phase processing medium to prevent the microparticles from agglomerating and to control the size of the solvent microdroplets in the emulsion. A preferred surfactant-dispersing medium combination is a 1 to 10 wt % poly(vinyl alcohol) in water mixture. The dispersion is formed by mechanical agitation of the mixed materials. An emulsion can also be formed by adding small drops of the active agent-wall forming material solution to the continuous phase processing medium. The temperature during the formation of the emulsion is not especially critical but can influence the size and quality of the microparticles and the solubility of the drug in the

continuous phase. Of course, it is desirable to have as little of the nucleic acids in the continuous phase as possible. The temperature must not be so low during processing as to make too viscous or solidify the solvent or processing medium. Nor should the temperature be so high as to evaporate too much medium or degrade the nucleic acids. Accordingly, the dispersion process can be conducted at any temperature which maintains stable operating conditions, preferably temperature being about 30°C to 60°C, depending upon the drug and excipient selected.

The dispersion which is formed by solvent extraction is a stable emulsion. From this dispersion the organic solvent immiscible fluid is partially removed in the first step of the solvent removal process. The solvent can easily be removed by well-known techniques such as heating, the application of a reduced pressure or a combination of both. The temperature used to evaporate solvent from the microdroplets is not critical, but should not be so high that it degrades the nucleic acids, nor should it be so high as to evaporate solvent at such a rapid rate to cause defects in the wall forming material. Generally, from 5 to 75%, and preferably 1 to 25%, of the solvent is removed in the first solvent removal step.

After the first solvent removal step, the dispersed microparticles in the solvent immiscible fluid medium are isolated from the fluid medium by any convenient means of separation. For example, the fluid can be decanted from the microparticle or the microparticle suspension can be filtered. Conventional combinations of separation techniques can be used if desired.

Following the isolation of the microcapsules from the continuous-phase processing medium, the remainder of the solvent in the microcapsules is removed by

extraction. In this second solvent removal step, the microcapsules can be suspended in the same continuous-phase processing medium used in the first solvent removal step, with or without surfactant, or in another liquid. The extraction medium removes the solvent from the microcapsules and yet does not dissolve the microcapsules. During the extraction, the extraction medium with dissolved solvent must be removed and replaced with fresh extraction medium. This is best done on a continual basis. The appropriate rate of extraction medium replenishment of a given process is easily determined by one skilled in the art. After the majority of the solvent has been removed from the microparticles, the microcapsules are dried by exposure to air or by other conventional drying techniques, such as vacuum drying, drying over a desiccant, or the like.

Another method of encapsulation is physical microencapsulation (U.S. Patent No.4,919,929). Physical microencapsulation techniques are characterized by the continuous envelopment of particles or droplets of a substance in a fluid film, as a melt or solution of the coating material, in an apparatus containing coaxially- or sequentially-spaced orifices. Thereafter, the fluid coating is hardened by a standard cooling technique or by solvent evaporation.

Among the physical methods for microencapsulation are those that involve the passage of liquid or solid core material through a liquid matrix material. The stream is disrupted by some means to cause the formation of liquid-coated droplets or particles, and the resulting particles are cooled or otherwise treated to solidify the shell material. For example, an aqueous solution of a substance to be encapsulated is aspirated into rapidly flowing stream of molten glycerol distearate, and the mixture is ejected through a fine

nozzle. On emergence from the nozzle, the liquid stream disintegrates into droplets, each consisting of an aqueous core surrounded by liquid wax. As these fall through air, the shells cool and solidify, and microparticles result. In another version of this process, the impelling force is supplied by a rotating member, which ejects the core material centrifugally through the shell-forming liquid.

The variations of these and other processes of microencapsulation are many. As is readily apparent to those skilled in the art, no one process nor any single set of conditions is applicable to all substances, but instead a useful process is chosen and the conditions optimized to achieve the desired results with a specific nucleic acid. Water soluble nucleic acids were encapsulated by the phase separation method. Lipid or organic soluble nucleic acids would be microencapsulated by the solvent extraction method.

In a preferred method of preparing microparticles containing a genetic substance, a phase separation technique is employed whereby a solution of the polymeric matrix material in a suitable organic solvent is prepared. To this solution is added the nucleic acids suspended or dissolved in water or as fine particles alone. A non-solvent for the polymeric matrix material is slowly added to the stirred dispersion causing the polymeric material to slowly precipitate around the nucleic acids forming microparticles. The microparticles are further hardened by the addition of a second non-solvent for the polymeric matrix material. The microparticles are then isolated by filtration and dried.

The microparticle products of Applicants' invention are usually made up of particles of a generally spherical shape, although sometimes the microcapsules

may be irregularly shaped. The microparticles can vary in size, ranging from submicron to millimeter diameters. Preferably, diameters less than 1 to 500 μm are desirable for nucleic acids formulations which allows administration of the microparticles with a standard gauge needle or other conventional methods. In other embodiments of the invention, the shaped nucleotide substance containing matrix material can assume forms other than microparticles such as rods, wafers, rectangularly shaped films or blocks. In each case the nucleic acid substance is distributed throughout the matrix material. The amount of nucleic acids dispersed throughout the matrix is an amount sufficient to elicit the desired therapeutic response as the entrapped nucleic acid is released by the implanted matrix material over an extended period of time. These shaped objects are particularly suitable for subcutaneous implantation into animals desired to be treated.

The amount of nucleic acids administered to the animal depends on the particular animal species, target gene sequence, illness, length of time of treatment, age of the animal, and amount of treatment desired.

Prior to administration to an animal or group of animals, the microparticles are suspended in an acceptable pharmaceutical liquid vehicle, and then the suspension is injected into the desired portion of the body of the animal.

The microparticles can be mixed by size or by type so as to provide for a delivery of nucleic acids to animals in a multiphasic manner and/or in a manner which provides different nucleic acids to the animal at different times, or a mixture of nucleic acids to the animal at the same time. Other biologically active agents commonly administered to animals may be blended with the nucleic acids formulation. For example,

antibiotics, antihelmintics, vaccines, or any desired active agent, either in microparticle form or in conventional, unencapsulated form may be blended with the nucleic acids and provided to an animal by the method of the invention.

METHOD-DELIVERY

In a preferred embodiment, the nucleic acids are administered to humans or animals by a single administration of the nucleic acid-loaded microcapsule, such that the microcapsules release the active gene in a constant or pulsed manner into the animalm, eliminating the need for repetitive injections. The micro-encapsulated gene can be injected or implanted or bombarded directly into the animal. These methods allow the direct insertion of genes into living animals. In the preferred method, the microparticle encapsulating the gene, its promoter and a gold, tungsten, platinum, ferrite, polystyrene, or latex particle is bombarded into the tissue. A method of particle bombardment is disclosed in U.S. Patent No. 4,945,050. The invention, however, is not limited to particle bombardment. Delivery of these microparticles can be effected by a variety of methods including direct injection, receptor mediated endocytosis, particle bombardment, implants (subcutaneous or intramuscular) or oral administration.

APPLICATIONS

The present invention relates to the genetic transformation of animal, plant and microbial cells as well as changes in gene expression with the introduction of new genetic material by transfer of nucleic acid (synthetic or natural), and slow release biodegradable polymeric microparticles containing the nucleic acid. The invention has a wide variety of applications, for example, in the breeding of plants and animals, the

understanding and treatment of diseases, and the production of protein.

Co-encapsulated compounds containing nucleic acids can be used for the expression of foreign genes, gene therapy, and the inhibition of gene activity. Various examples describing how this technology could be applied are:

1. HUMAN GENE THERAPY

Muscular dystrophy can be treated by implants of the encapsulated dystrophin gene into the muscle tissue below the fascia. Uptake of the gene can occur by transport through the sarcoplasmic reticulum or through the cut ends of the muscle. Direct injection or bombardment of the encapsulated gene can also allow the facilitated uptake of the gene into muscle cells. Knockout of the endogenous defective gene would improve the effects of the newly expressed gene. Uptake can be by use of ligands bound to the microencapsulated gene.

2. CANCER TREATMENT

Treatment of metastatic cancers can be accomplished by inserting genes for cytokines or genes for killing the cancerous cells. Direct insertion of genes into tumor infiltrating lymphocytes have now been accomplished (Fitzpatrick-McElligott, Bio/Tech. 10:1036-1040 (1992). An encapsulated gene construct containing a promoter/regulator enhancer region and coding region will be inserted into solid tumors. Tumor infiltrating lymphocytes (TIL) cells receiving the cytokine gene will then circulate to other metastatic sites. The protein produced intracellularly can then affect the cancerous cells at this location. After gene insertion in culture the cells can be grown and reinfused into the body. The encapsulation process will serve to protect the gene from degradation.

3. TRANSGENIC ANIMAL PRODUCTION

Animals can be made to express foreign proteins or altered natural products by introduction of new genetic information. This method offers a significant advantage over conventional production of transgenic farm animals. With this method, a pregnant cow could be injected in vivo with a gene encoding a recombinant protein, driven by a mammary gland-specific promoter. The gene would be taken up in the developing epithelial cells and expressed at the time of lactation. This process takes only a few weeks whereas expression of recombinant proteins in cows by conventional transgenic technology takes a minimum of 30 months.

4. GENETIC IMMUNIZATION

The following example demonstrates a novel method for inserting genes into living animals (in vivo studies) in order to express a foreign protein which in turn would elicit an antigenic response. The encapsulated gene/promoter sequence for the coat protein for the HIV virus can be inserted into cells allowing the production of the antigenic portion of the virus without the actual virus. This eliminates the risk of retroviral replication. Several publications now acknowledge that retroviral vectors pose considerable risk. Although composed of recombination-incompetent viruses, these vectors can be reactivated and cause infection. (see Temin, Human Gene Therapy 1:111-123 (1990)) Retroviral vectors have potential problems, oncogenesis, pathogenesis, and homologous recombination with helper sequences which can be used to propagate the vectors. This change can lead to the escape of competent infectious virus.

5. GENETIC IMMUNIZATION

DNA containing the sequence for hepatitis B virus surface antigen driven by the actin promoter is

microencapsulated by the phase separation method. 25 mg of plasmid DNA is encapsulated in 65:35 polylactide and polyglycolide (PLGA) in 1 g and 2.5 g batches at 5% and 1% loading, respectively. The microspheres preparation is then injected into the breast muscle of chickens. At 1, 2, 4, and 8 weeks, blood samples are taken and the titer of anti-HBV antibody determined. The cell mediated immune response against HBVSA is evaluated by analysis of lymphokine secretion from chicken WBC stimulated in the presence of macrophages and antigen.

6. GENE KNOCKOUT VIA HOMOLOGOUS RECOMBINATION

A DNA construct encoding a replacement gene for a defective cellular gene responsible for an inborn error of metabolism could be engineered for integration via homologous recombination. The construct could then be conjugated to a ligand that would target its uptake to the cell in which the defective gene is expressed. The conjugate could be microencapsulated and injected into a patient to provide, over time, enough copies of the new gene to enter the target cell and knock out the defective gene, replacing it with the correct sequences. A possible example of how knockout/replacement therapy might be of therapeutic use is in hemophilia A. Hemophilia A is an X-chromosome linked clotting disorder caused by a defect in the blood clotting factor VIII gene. A new factor VIII gene might be used to replace a defective sequence by injection of the microencapsulated factor VIII gene/asialoglycoprotein conjugate i.p. There it would be absorbed through the portal circulation and transported to the liver for recombination and expression.

EXAMPLES

The present invention will now be described by reference to specific examples which are meant to be

illustrative only and are not intended to limit the invention.

Throughout the disclosure the following meanings are intended: "sec" refers to seconds, "min" refers to minutes, "h" refers to hours, "d" refers to days, "mL" refers to milliLiters, and "g" refers to grams.

EXAMPLE 1

MICROENCAPSULATION OF PLASMID DNA IN BIODEGRADABLE POLYMERS

The following three examples make use of plasmid DNA, and tungsten microcarrier particles microencapsulated by the phase separation method. Two plasmids were used in these experiments; (1) pMH40, (E. I. du Pont de Nemours and Company, Agricultural Products, Wilmington, DE) which is 7.3 kilobases (kb) in length and contains the ß-glucuronidase gene driven by the cauliflower mosaic virus promoter with an SV-40 virus 3' slice site, and (2) The plasmid pRC/CMV/ß-gal, which is 11.6 kilobases (kb) in length and contains the ß-galactosidase gene and the coding region for neomycin phosphotransferase II which confers resistance to the antibiotics kanamycin and G418 (InVitrogen, San Diego, CA). The gene expression is driven by the cytomegalovirus promoter. Neither of these plasmids contain complete viral genomes and neither are infectious.

For microencapsulation plasmid pMH-40 DNA [500 µg or 2000 µg in 500 µL 10 mM tris (pH7.4), 1 mM EDTA (TE) buffer] or plasmid pRC/CMV/Bgal DNA [500 µg in 500 µl 10 mM tris (pH 7.4), 1 mM EDTA (TE) buffer] plus 500 µl of 50 mg/mL Herring sperm DNA (Boehringer Mannheim, Indianapolis, IN) dissolved in TE was incubated in a shaking water bath at 65°C for 30 min to promote mixing. The biodegradable polymer used for encapsulation contained the monomers lactide and glycolide in a ratio

of 65:35 (dl-PLGA) (Medisorb Technologies Int., Cincinnati, OH) was weighed into a 50 mL glass screw-cap tube and dissolved in 31.7 g of ethyl acetate. To this was added, 0.75 g of M-17 tungsten microcarrier particles (Biorad, Richmond, CA), the contents were vigorously agitated and then poured into a 300 mL water jacketed reaction vessel cooled to 0°C. An additional 43.7 g of ethyl acetate was added to the reactor and the mixture was probe sonicated (Tekmar Model TM375) as 1 mL of DNA solution was slowly added using a 1cc syringe and 18 gauge needle. After 30 sec of sonication, 74 g of 360 fluid 1000 cs silicon oil (Dow Corning, Ithaca, NY) was added to the reactor over 2 min and this mixture was then immediately quenched by stirring at room temperature in 2.5 liters of heptane (Chempure M138 KBJS). After 3.5 h the solid material was collected on a 0.2 μm filter, washed with heptane and dried in a vacuum oven for at least 3 d. These microspheres are extremely sensitive to moisture and to temperatures above 30°C and are therefore stored desiccated at 4°C.

Light microscopic pictures (Fig. 1) show the fluorescently labelled DNA indicated by black arrows can be microencapsulated. The tungsten core added to the microparticles are designated by white arrows. Scanning electron micrographs (Fig. 2) show the size distribution of the microparticles after encapsulation to range in size from >1 μm to <250 μm. The surface of the particles appear smooth with the particles sticking to each other. The action of bombardment and impact against the screen break up the particles into smaller components. The results show that DNA can be micro-encapsulated in a size effective for cellular and tissue insertion. The dense tungsten core adds the necessary density for particle bombardment. However, the invention is not limited to particle bombardment and

different sizes and shapes can be constructed for direct injection and implants.

EXAMPLE 2

RELEASE AND IDENTIFICATION

OF PLASMID DNA AFTER MICROENCAPSULATION

This example shows the release of DNA from the microcapsules over time. The microencapsulated DNA was recovered by dissolution of 50 mg of the microcapsules in 500 µL of 24:1 chloroform/isoamyl alcohol with simultaneous extraction of the DNA into TE buffer at room temperature. The DNA in the aqueous phase was precipitated by the addition of one tenth volume of 3M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol at -20°C. The integrity of the recovered DNA was analyzed by ethidium bromide/agarose gel electrophoreis and visualized by UV illumination. The identity of the plasmid DNA recovered from the microspheres by this method was verified by digestion with restriction endonuclease Eco R1. The plasmid DNA (10 µg) was cut overnight at 37°C with 20 units of Eco R1. Plasmid pMH40 was analyzed on a 0.7% agarose gel, stained with ethidium bromide and photographed. ECO-R1 - cut pMH 40 yield two fragments of the predicted size; 3.3 kb and 4.0 kb in length. Plasmid pRC/CMV/ß-gal has 2 Eco R1 restriction sites and 1 Bam HI site. The plasmid DNA (10 µg) was cut overnight at 37°C with 50 units of Eco R1 or Bam HI. After digestion, the cut DNA was analyzed on a 0.7% agarose gel, stained with ethidium bromide and photographed. Eco R1-cut DNA yielded two fragments of the predicted length and Bam HI cut DNA yielded one restriction fragment.

Controlled release of the DNA *in vitro* was analyzed. 50 mg of Batch 233 microspheres or control unloaded 65:35 microspheres were suspended in 25 mL of TE buffer and incubated at 37°C in a shaking water bath.

Samples (500 µl) were taken at 1, 3, 6, 24, 48, and 72 h, pooled precipitated by the addition of one tenth volume of sodium acetate (pH 5.2) and frozen at -20°C. The precipitated DNA was pelleted by centrifugation at 2000 g at 4°C for 20 min and the resulting pellet washed in 70% ETOH at -20°C to remove residual salt, and dried in a DNA Speedvac for 10 min. The pellet was resuspended in TE and quantitated by UV spectrophotometry. DNA recovered from the in vitro dissolution analysis containing pMH40 was analyzed by polymerase chain reaction. DNA was mixed with 3' and 5' primers, 2.5 units of Amplitaq (Perkin-Elmer, Cetus, Norwalk, CT), and 2 mM dNTPs in a buffer containing 10 mM tris (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, and subjected to 10, 15, 20, and 25 PCR cycles using a DNA thermal cycler model 480 (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of 1 min at 94°C, 1 min at 42°C, and 3 min at 74°C. Analysis of the PCR-amplified DNA by agarose gel electrophoresis and ethidium bromide staining showed that a single 625 bp DNA fragment was amplified and that the DNA was released over time (Fig. 3). Thus, these results indicate that the integrity of the DNA remains intact during the microencapsulation and release process.

EXAMPLE 3

GENE EXPRESSION IN PLANT CELLS AFTER DELIVERY OF ENCAPSULATED DNA

Microencapsulated DNA with a tungsten core of .5 µm was bombarded into the tissue. The DNA consisted of 75% herring sperm DNA with 25% ß-glucuronidase plasmid (pMH40). Tungsten/DNA-loaded microspheres (5 mg) were prepared by suspension in 0.075 mL ice cold 70% ETOH and sonication on ice. pMH-40 DNA was diluted to 1 µg/µL precipitated onto 1.0 µm tungsten microcarriers as previously described. Briefly, 60 mg of microcarriers

were washed in 1 mL of 100% ethanol, sonicated for 30 sec, spun down at 12,000 g and washed with 1 mL of sterile distilled water, sonicated and spun down again. The supernatant was decanted and 0.5 mL of sterile distilled water was added to the microcarriers. Then, 25 µL of the microcarrier suspension was placed in a new sterile 1.5 mL microcentrifuge tube and to this was added 10 µL of pMH40 plasmid DNA in TE, 25 µL of 2.5M $CaCl_2$ and 10 µL of 0.1M spermidine (Sigma, St. Louis, MO) while continuously vortexing. After 10 min of room temperature incubation, the microcarriers with the DNA precipitated on them were pelleted at 10,000 g for 2 min and the supernatant removed. The pellet was resuspended in 0.25 mL 100% ethanol, briefly sonicated, repelleted at 10,000 g and resuspened in 60 µL of 100% ethanol.

Bombardment of cauliflower stems was performed by spreading 15 µL of the prepared particles on to a Kapton macrocarrier disc and allowing the ethanol to evaporate. Bombardment was conducted under 27 in. Hg vacuum and at 2000 p.s.i. rupture disk pressure using the Biolistic PDS-1000-HE particle delivery system (BioRad, Hercules, CA).

ß-glucuronidase (GUS) gene expression was analyzed in bombarded cauliflower. The tissue was incubated at room temperature for 18 h under constant illumination followed by 6 h of darkness and stained for 24 h in the dark at 37°C. The assay solution contained 0.1 nM Na_2HPO_4 pH7.0, 0.5 mM K_3 Fe[CN]$_6$, 0.5 mM K_4 Fe[CN]$_6$ $3H_2O$, 10 mM Na_2 EDTA, 0.5 mg/mL X-glu sodium salt (Biosynth, Staad, Switzerland).

The results (Fig. 4) shows delayed expression of the transgene activity in cauliflower four days after bombardment with microencapsulated DNA. The micro-capsules contained the ß-glucuronidase marker gene. Control pieces of tissue without bombardment or after

bombardment without DNA did not show any blue staining. Thus an exogenous encapsulated gene was delivered to plant cells and was capable of causing delayed transformation of the tissue. Thus, gene expression in plants was demonstrated by use of the slow release of the reporter gene ß-glucuronidase inserted into intact cauliflower.

EXAMPLE 4

STABLE GENE EXPRESSION IN ANIMAL CELLS WITH A MICROENCAPSULATED GENE

Plasmid pRC/CMV/Bgal and tungsten microcarrier particles were microencapsulated by the phase separation method. The conditions for microencapsulation were performed as detailed in Example 1, except for the following. The concentration of DNA was 500 µg in 500 µL of 10 mM tris pH 7.4. The ethyl acetate was added was at a weight of 44.4 g to the reactor. The silicon oil 67.9 g instead of 74 g was added to the reactor. For bombardment, pRC/Bgal DNA was diluted to 1 µg/µL precipitated onto 1.0 µm tungsten microcarriers as previously described. Briefly, 60 mg of microcarriers were washed in 1 mL of 100% ETOH, sonicated for 30 sec, spun down at 12,000 g and washed with 1 mL of sterile distilled water. The supernatant was discarded and 0.5 mL of sterile distilled water was added. Then, 25 µL of the microcarrier suspension was placed in a new 1.5 mL microcentrifuge tube and to this was added 10 µL of pMH40 plasmid DNA in TE, 25 µl of 2.5M Cacl and 10 µL of 0.1M spermidine (Sigma, St. Louis, MO.) while continuously vortexing. After 10 min at 4°C, the microcarriers with the DNA precipitated on them were pelleted at 10,000 g for 2 min and the supernatant removed. The pellet was resuspended in 0.25 mL 100% ETOH, briefly sonicated, repelleted at 10,000 g and resuspended in 60 µL of 100% ETOH.

Tungsten/DNA-loaded microspheres (5 mg) were prepared by suspension in 0.075 mL ice cold 70% ETOH and sonication on ice. Bombardment of confluent CHO cells was performed by spreading 15 µL of the prepared particles on to a Kapton macrocarrier disc and allowing the ETOH to evaporate. Bombardment was conducted under 15 in. Hg vacuum and at 1350 p.s.i. gas pressure using the Biolistic PDS-1000-HE particle delivery system (BioRad, Hercules, CA). After bombardment, the cells were put back into culture media and grown for two days under standard conditions without G-418 antibiotics. After 2 d the cells were selected in media containing G-418 for 6 to 8 weeks. Live colonies of transformed cells (Fig. 5) were observed in the bombarded cultures after 6 weeks of growth in G418 media. Control cultures bombarded with microcapsules containing only herring sperm DNA and tungsten showed no live colonies. The results showed that animal cells in culture can be stably transformed when microencapsulated genetic material is inserted into the cells.

ß-galactosidase activity in bombarded and selected CHO cells was analyzed. Staining for ß-galactosidase enzyme activity was performed by fixation of cells on Petri plates for 15 min in 0.05% glutaraldehyde in phosphate buffered saline (PBS), the fixative was removed by three rinses with PBS and the cells were stained by the addition of the X-gal solution and incubated for 2-6 h. The X-gal solution consisted of 10mM Na PO_4, 3mM K_3 Fe[CN]$_6$, 3mM K_4 Fe[CN]$_6$ $3H_2O$, 1 M $MgSO_4$, 150mM NaCl, 1mM $MgCl_2$, 0.2% X-gal. Further evidence for the gene insertion is the blue color seen after staining for the ß-galactosidase activity (Fig. 5).

Integration of the ß-galactosidase gene was determined by PCR analysis of genomic DNA purified from

G-418 resistant CHO cell clones (Fig. 6). Clones (Fig. 5) were produced by bombardment with batch 231 microspheres as described above, dilution and plating in 96 well, flat bottom tissue culture plates in media containing G-418 for 3-4 weeks and expanded. DNA was isolated from the clones using the Stratagene DNA isolation kit. PCR was performed as above using 2 µg of clone DNA per reaction. Results show that the ß-galactosidase gene has integrated in genomic DNA of the clones (Fig. 6).

EXAMPLE 5

ENCAPSULATION OF CONJUGATES AND GENE TRANSFER BY RECEPTOR-MEDIATED ENDOCYTOSIS

Gene transfer may be accomplished by the receptor - mediated endocytosis pathway, for example using transferrin-polylysine or polylysine-asialoglycoprotein conjugated to DNA. The strategy of gene transfer by this method uses bifunctional molecular conjugates consisting of a cognate moiety for a cell surface receptor that is linked to a DNA-binding moiety. For some target cells, however gene transfer by this method is limited. This limitation is due to degradation by various enzymes, lysosomal, proteases, nucleases. To enhance DNA protection from enzymatic degradation, we propose to encapsulate the conjugate and its DNA. One additional advantage of the proposed encapsulation is the ability to include, in the microcapsules, selected lysosomatropic agents which would degrade lysosomes. Such lysosomatropic agents include chloroquine (Zenke, et al., Proc. Natl. Acad. Sci. 87:3655-3659 (1990)), and adenoviruses (Curiel et al., Proc. Natl. Acad Sci. 88:8850-8854 (1991)).

To prepare the transferrin-poly(L-Lysine)-DNA complexes, Applicants would follow the method of Cotten et al. Proc. Natl. Acad Sci., 87:4033-4037 (1990)) and

Zenke, et al. Proc. Natl. Acad. Sci., 87:3655-3659 (1990)). The specific ligation will be accomplished through modification of the transferrin carbohydrate moiety. The DNA plasmid pRSVL containing the *Photinus pyralis* luciferase gene under the control of the Rous sarcoma virus long terminal repeat enhancer/promoter (DeWet et al. Mol. Cell Biol., 7:725-737 (1987) will be used as a reporter gene. Conjugate-DNA complexes will be prepared by dilution of 6 µg of pRSVL DNA in 350 µL of HBS (150mM NaCl/20 mM Hepes, pH 7.3) followed by addition to 12 µg of hTfpL190B diluted in 150 µL of HBS. Complexes will be allowed to form for 30 min at room temperature. Adenovirus dl312, a replication-incompetent strain deleted in the Ela region (Jones and Shenk, Proc. Natl. Acad. Sci., 76:3665-3669 (1979)) will be prepared as described by Curiel et al., (Proc. Natl. Acad Sci., 88:8850-8854 (1991)). Chloroquine can be included in the microcapsules at a concentration of 100 µM.

Microencapsulation of the DNA-conjugates will be performed as detailed in Example 1 except for the use of the plasmid pRSVL-luciferase DNA conjugate at a concentration of 500 µg in 500 µL of 10mM tris pH7.4. Either adenovirus dl312 or chloroquine (100 µM) can be included during the encapsulation. Evaluation of the effect of the microencapsulated DNA-conjugates can be performed in vitro on cell lines such as the human leukemic cell line (Cotten et al., Proc. Natl. Acad. Sci., 87:4033-4037 (1990)) or HeLa cells grown according to established conditions (Curiel et al., Proc. Natl. Acad. Sci., 88:8850-8854 (1991)). Microencapsulated DNA conjugates will be added directly to the cells in culture and incubated at 37°C for 48 and 72 h. After incubation the cells will be harvested for luciferase gene expression. These results will be compared to

results on gene expression using DNA-conjugates without microencapsulation.

Polylysine-asialoglycoprotein conjugated to DNA (Wu and Wu, (J. Biol. Chem., 263:14621-14624 (1998)) will also be used to target liver cells in vivo. The procedure for conjugation of DNA (pSV2 CAT plasmid) to polylysine -asialoglycoprotein is described by Wu and Wu, (J. Biol. Chem., 263:14621-14624 (1988)). The DNA conjugate will be prepared by covalently linking poly-L-lysine to the galactose-terminal (asialo-)glycoprotein, asialoorosomucoid (AsOR). Poly-L-Lysine (Sigma) will be coupled to AsOR in a 2:1 molar ratio using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce Chem. Co.) using the procedure of Wu et al. (J. Biol. Chem., 264:16985-16987 (1989)). Complexes with DNA will be formed at a conjugate to DNA molar ratio 2:1 as determined by Wu and Wu, (J. Biol. Chem., 263:14621-14624 (1988)). Samples will be incubated for 1 h at 25°C and then dialyzed for 24 h against 0.15 M saline through membranes with a molecular weight limit of 3500 (Spectrum Medical Industries CA.). After dialysis all samples will be filtered through a 0.2 μm membrane (Millipore Corp) to ensure that the complexes do not contain precipitates.

Encapsulation of the conjugate will be performed as previously described in Example 1. The molecular conjugates and the lysosomatropic agents are encapsulated by the phase separation method in 1 gram of polylactide and polyglycolide copolymer (PLGA). Biodegradable polymer containing a mixture of 65% poly-lactic and 35% poly-glycolic acids (dl-PLGA) (Medisorb Technologies Int., Cincinnati, OH) is weighed into a 50 mL glass screw-cap tube and dissolved in 30 g of ethyl acetate and then poured into a 300 mL water jacketed reaction vessel cooled to 0°C. An additional

45 g of ethyl acetate is added to the reactor and the mixture probe sonicated (Tekmar Model TM375) as 1 mL of DNA solution containing 25 mg of the conjugate in 10 mM Tris pH 7.4, 0.1 mM EDTA (TE) buffer is slowly added using a 1cc syringe and 18 gauge needle. After 30 sec of sonication, 75 g of 360 fluid 1000 cs (Dow Corning, Ithaca, NY) silicon oil is added to the reactor over 2 min and this is then immediately quenched by stirring at room temperature in 2.5 L of heptane (Chempure M138 KBJS). After 3.5 h, the solid material is collected on a 0.2 μm filter washed with heptane and dried in a vacuum oven for at least 3 d. The microcapsules range in size from greater than 1 μm to less than 250 μm. Microspheres are extremely sensitive to moisture and temperatures above 30°C and are stored desiccated at 4°C.

In vivo uptake can be measured after injection intramuscularly or intravenously into Sprague-Dawley rats with 1 mg of encapsulated pSV2 CAT DNA in a sterile saline solution. Tissue samples can be monitored for CAT activity at 24-72 h using the procedure described by Wu et al. (J. Biol. Chem., 264: 16985-16987 (1989)).

An improvement in gene expression is predicted from the use of these procedures because of the protection of the DNA and conjugate within the microcapsules. Slow release of the DNA will prolong the effect of the gene transfer and allow gene expression over an extended period of time.

EXAMPLE 6

PRODUCTION OF RECOMBINANT PROTEIN IN CHICKEN EGG WITH ENCAPSULATED CONJUGATED DNA

Plasmid DNA is amplified in bacteria, purified and linearized by enzymatic digestion with restriction endonuclease. The linear plasmid containing the gene encoding human growth hormone (hGH), driven by the

ovalbumin promoter is conjugated to chicken insulin by cross-linking with disuccinimidyl suberate using the method of Huckett et al. (Biochem. Pharmacol., 40:253-263 (1990)). This molecular conjugate is encapsulated by the phase separation method in 1 g of polylactide and polyglycolide copolymer (PLGA). Biodegradable polymer containing a mixture of 65% poly-lactic and 35% poly-glycolic acids (dl-PLGA) (Medisorb Technologies Int., Cincinnati, OH) is weighed into a 50 mL glass screw-cap tube and dissolved in 30 g of ethyl acetate and then poured into a 300 mL water jacketed reaction vessel cooled to 0°C. An additional 45 g of ethyl acetate is added to the reactor and the mixture probe sonicated (Tekmar Model TM375) as 1 mL of DNA solution containing 25 mg of the conjugate in 10 mM Tris pH 7.4, 0.1 mM EDTA (TE) buffer is slowly added using a 1cc syringe and 18 gauge needle. After 30 sec of sonication, 75 grams of 360 fluid 1000 cs silicon oil (Dow Corning, Ithaca, NY) is added to the reactor over 2 min and this is then immediately quenched by stirring at room temperature in 2.5 liters of heptane (Chempure M138 KBJS). After 3.5 h, the solid material is collected on a 0.2 µm filter washed with heptane and dried in a vacuum oven for at least 3 d. The microcapsules range in size from greater than 1 µm to less than 250 µm. These microspheres are extremely sensitive to moisture and temperatures above 30°C and are therefore stored desiccated at 4°C.

A bolus of microspheres suspended in CMC injection vehicle is injected i.p. into 25 week old leghorn laying hens and the level of growth hormone is determined in each egg by ELISA. Applicants predict that the conjugated plasmid-insulin complex will be released into the peritoneal cavity and home to the basal surface of the oviductal epithelial cells, where it will be taken

up by these cells and the hGH gene will be expressed. The hormone will be secreted into the oviduct lumen and incorporated into the chicken egg.

EXAMPLE 7

GENETIC IMMUNIZATION

The production of an immune response against a foreign antigen usually requires purification of the protein, which is then injected into the animal. The isolation of enough protein is difficult and time-consuming. Applicants directly insert encapsulated genes either by injection or particle bombardment into skin or muscle. This constitutes a unique method for vaccination or antibody production.

Innoculation of mice (ICR strain) using the human growth hormone (hGH) is done using microcapsules containing the human growth hormone gene under the transcriptional control of either the human ß-actin promoter (Leavitt et al., Molec. Cell Biol., 4:1961-1969 (1984)) or the cytomeglovirus (CMV) promoter (Boshart et al., Cell, 41:521-530 (1985)). The microcapsules for particle bombardment also contain inert particles of dense material preferably of gold, or tungsten. The dense particle will improve the momentum and allow penetration into the cells of the animal. Example 1 describes the method for encapsulation with inert particles, except that in this example Applicants use the human growth hormone gene and either the human ß-actin or the CMV promoter. The microcapsules are propelled by the hand-held Biolistic system (see Tang et al., Nature, 356:152-154 (1992)). Alternatively, the bolus of encapsulated microcapsules containing the hGH gene is injected directly into the muscle of the mice at several sites.

Production of antibodies directed against hGH is monitored by assaying sera from tail-bleeds for the

capacity to immunoprecipitate ^{125}I-labelled hGH. Applicants measure the amount of antibody to hGH by incubating 1 µL of sera with 1 µL ^{125}I-labelled hGH (DuPont-NEN, 84-88 µCi mL^{-1}; 113-116 µCi Mg^{-1}) for 1 h at room temperature. Protein A agarose beads (4 µL Pierce) will be added and the slurry incubated for 12-18 h at 4°C. The beads will be pelleted by centrifugation and washed thoroughly with phosphate buffered saline (PBS) before determining the counts per min (cpm) retained. Values are normalized to nanograms of hHG precipitated per µL of serum. Antibodies against hGH in the sera of genetically immunized mice are detected by western blot analysis (see Tang et al., Nature, 356:152-154 (1992)).

The present invention is not to be limited to the particular embodiment or examples disclosed above, but embraces all such modified forms thereof as come within the scope of the following claims.

WHAT IS CLAIMED:

1. A microparticle composition suitable for the controlled release of a nucleic acid to a target cell, the microparticle comprising:

(a) a nucleic acid comprising synthetic or natural DNA or RNA exogenous or native to a target cell, the nucleic acid conjugated by way of chemical bonds with promoting material which promotes the uptake or transport to the nucleus, or expression of the nucleic acid in the cell, the molecules selected from the group consisting of glycoproteins, lipoproteins, nucleoproteins and peptides, hormones, antibodies, growth factors, nucleic acid binding factors, proteinaceous cellular ligands, glycolipids, peptidoglycans, lectins, fatty acids, phospholipids, glycolipids, triglycerides, steroid hormones, cholesterol, single stranded or double stranded RNA, single stranded or double stranded DNA, and intercalating agents, the nucleic acid present in an amount of about 0.0001 wt % to 50 wt % based on the parts of nucleic acid per weight of an encapsulating polymeric matrix of element (b);

(b) a biocompatible; biodegradable polymeric matrix encapsulating the nucleic acid of element (a), the polymeric matrix selected from the group consisting of poly-d, L-lactic acid, poly-L-lactic acid, polyglycolic acid, copolymers of mixed d,L-lactic acid and glycolic acid, copolymers of L-lactic acid and glycolic acid, copolyoxalates, polycaprolactone, poly(lactic acid-caprolactone), poly(glycolic acid-caprolactone), casein, albumin, and waxes; and

(c) the microparticle ranging in diameter from 1 to 500 microns.

2. A microparticle composition suitable for the controlled release of a nucleic acid to a target cell, the microparticle comprising:

(a) a nucleic acid comprising synthetic or natural DNA or RNA exogenous or native to a target cell;

(b) a promoting material which promotes the uptake of the nucleic acid of element (a) into the target cell or the transport of the nucleic acid of element (a) to the nucleus of the target cell, the material selected from a group consisting of glycoproteins, lipoproteins, nucleoproteins, peptides, hormones, antibodies, growth factors, nucleic acid binding factors, proteinaceous cellular ligands, glycolipids, carbohydrates, sphingolipids, peptidoglycans, lectins, fatty acids, gangliosides, phospholipids, triglycerides, cholesterol, single-stranded or double-stranded RNA, single-stranded or double-stranded DNA, and intercalating agents;

(c) a biodegradable, biocompatible polymeric matrix, within which encapsulating matrix the nucleic acid of element (a) and the promoting material of element (b) coexist, the polymeric matrix selected from the group consisting of poly-d, L-lactic acid, poly-L-lactic acid, polyglycolic acid, copolymers of mixed d,L-lactic acid and glycolic acid, copolymers of L-lactic acid and glycolic acid, copolyoxalates, polycaprolactone, poly(lactic acid-caprolactone), poly(glycolic acid-caprolactone), casein, albumin, and waxes;

(d) the nucleic acid of element (a) present in an amount of about 0.0001 wt % to 50 wt % based on parts of nucleic acid per weight of the polymeric matrix of element (c);

(e) the microparticle ranging in diameter from 1 to 500 microns.

3. The microparticle composition of Claim 1 further comprising an inert particle as the core of the microparticle.

4. The microparticle composition of Claim 2 wherein the inert particle is tungsten, gold, platinum, ferrite, polystyrene, or latex.

5. A method for preparing nucleic acids in a size effective for cellular or tissue insertion to effect gene expression in non-human animals and plants comprising forming a controlled-release, biocompatible, biodegradable microparticle comprising:

(a) a nucleic acid comprising synthetic or natural DNA or RNA exogenous or native to a target cell, the nucleic acid conjugated by way of chemical bonds with promoting material which promotes the uptake or transport to the nucleus or expression of the nucleic acid in the cell, the molecules selected from the group consisting of glycoproteins, lipoproteins, nucleoproteins and peptides, hormones, antibodies, growth factors, nucleic acid binding factors, proteinaceous cellular ligands, glycolipids, peptidoglycans, lectins, fatty acids, phospholipids, glycolipids, triglycerides, steroid hormones, cholesterol, single stranded or double stranded RNA, single stranded or double stranded DNA, and intercalating agents, the nucleic acid present in an amount of about 0.0001 wt % to 50 wt % based on the parts of nucleic acid per weight of an encapsulating polymeric matrix of element (b);

(b) a biocompatible, biodegradable polymeric matrix encapsulating the nucleic acid of element (a), the polymeric matrix selected from the group consisting of poly-d, L-lactic acid, poly-L-lactic acid, polyglycolic acid, copolymers of mixed d,L-lactic acid and glycolic acid, copolymers of L-lactic acid and

glycolic acid, copolyoxalates, polycaprolactone, poly(lactic acid-caprolactone), poly(glycolic acid-caprolactone), casein, albumin, and waxes; and

(c) the microparticle ranging in diameter from 1 to 500 microns.

6. A method for preparing nucleic acids in a size effective for cellular or tissue insertion to effect gene expression in animals and plants comprising forming a controlled-release, biocompatible, biodegradable microparticle comprising:

(a) a nucleic acid comprising synthetic or natural DNA or RNA exogenous to a target cell;

(b) a promoting material which promotes the uptake of the nucleic acid of element (a) into the target cell or the transport of the nucleic acid of element (a) to the nucleus of the target cell, the promoting material selected from a group consisting of glycoproteins, lipoproteins, nucleoproteins, peptides, hormones, antibodies, growth factors, nucleic acid binding factors, proteinaceous cellular ligands, glycolipids, carbohydrates, sphingolipids, peptidoglycans, lectins, fatty acids, gangliosides, phospholipids, triglycerides, cholesterol, single-stranded or double-stranded RNA, single-stranded or double-stranded DNA, and intercalating agents;

(c) a biodegradable, biocompatible polymeric matrix, within which encapsulating matrix the nucleic acid of element (a) and the promoting material of element (b) coexist, the polymeric matrix selected from the group consisting of poly-d, L-lactic acid, poly-L-lactic acid, polyglycolic acid, copolymers of mixed d,L-lactic acid and glycolic acid, copolymers of L-lactic acid and glycolic acid, copolyoxalates, polycaprolactone, poly(lactic acid-caprolactone),

poly(glycolic acid-caprolactone), casein, albumin, and waxes;

(d) the nucleic acid of element (a) present in an amount of about 0.0001 wt % to 50 wt % based on parts of nucleic acid per weight of the polymeric matrix of element (c);

(e) the microparticle ranging in diameter from 1 to 500 microns.

7. The method of Claims 5 and 6, wherein said polymeric matrix containing the nucleic acids is coated with a promoting material which promotes the uptake of the nucleic acid into the cell or the transport of the nucleic acid to the nucleus, the promoting molecule selected from a group consisting of proteins, intercalating agents, nucleic acids, lipids, or carbohydrates.

8. A method for the controlled delivery of an exogenous or native gene into the cells of plants or animals to effect gene expression, the method comprising:

(a) encapsulating a genetic construct exogenous or native to a target cell within a biocompatible, biodegradable polymer matrix, the encapsulating step forming a microparticle composition;

(b) containing the microparticle composition of step (a) in a dosing device suitable for delivery of the microparticle to a target cell;

(c) discharging the microparticle composition of step (a) into a plant or animal from which site the genetic construct contained within the microparticle composition can interact with the nucleus of a target cell upon degradation of the polymer matrix.

9. The method of Claim 8, wherein the microparticle composition is discharged into a plant or

animal parenterally, topically, orally or by particle delivery method.

FIG. 1

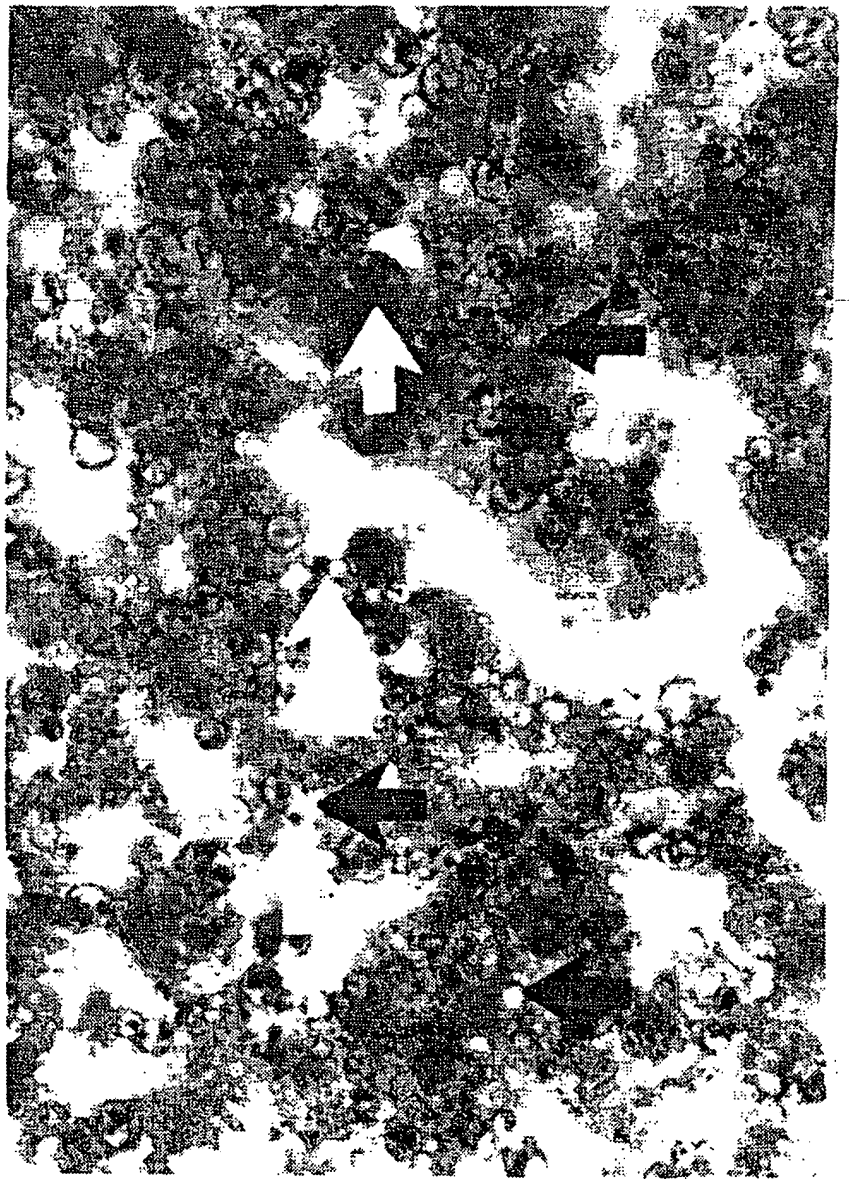


FIG. 2

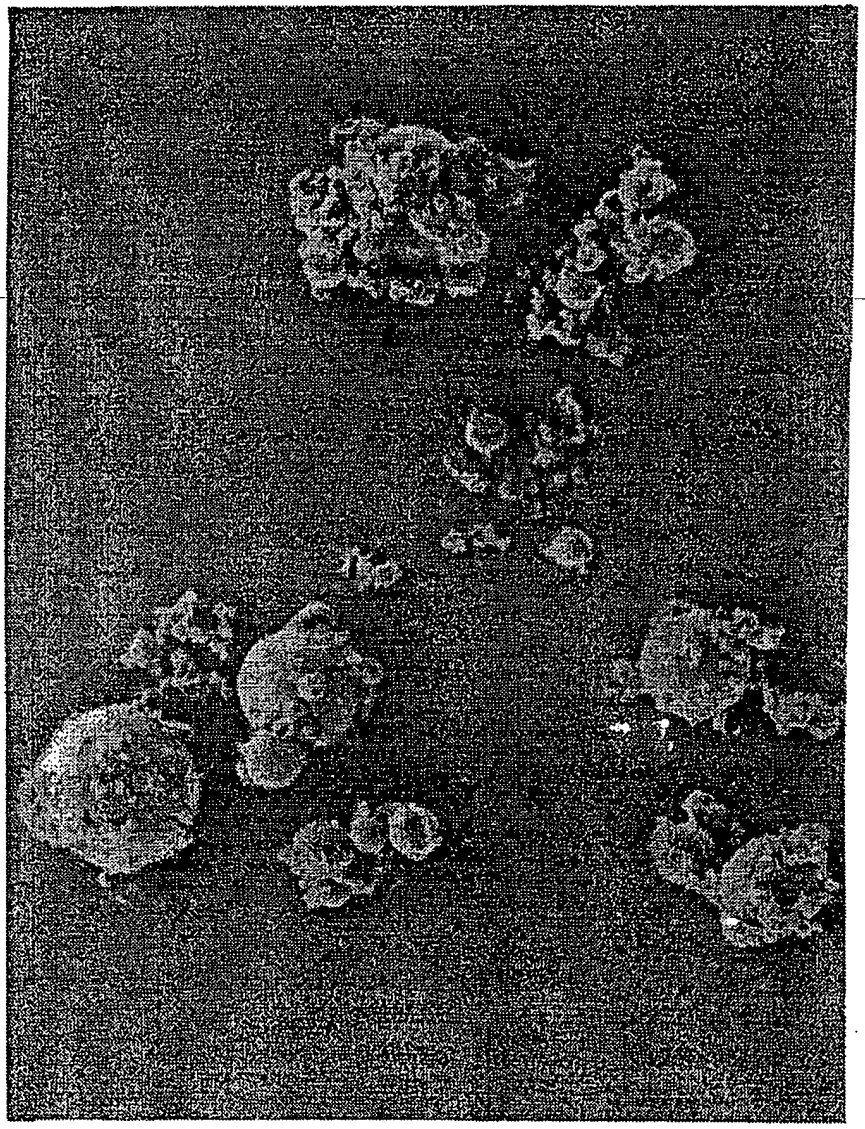


FIG. 3

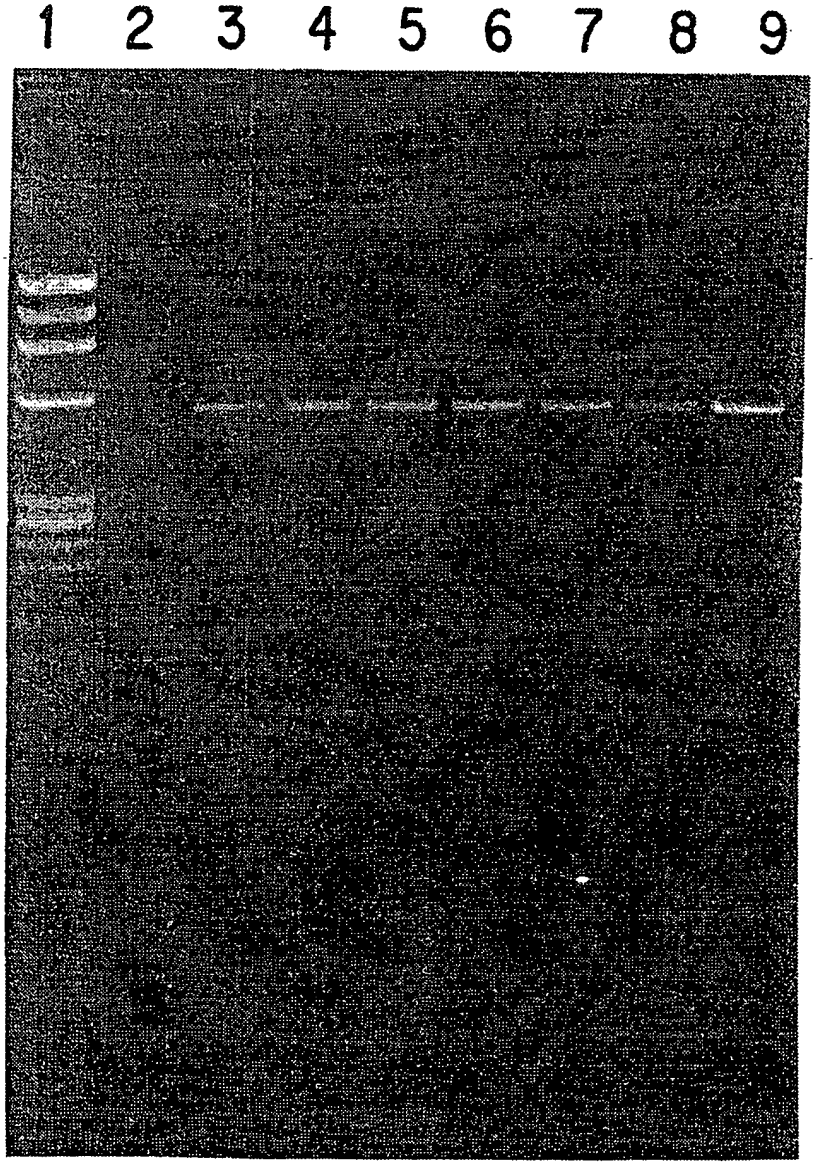


FIG.4

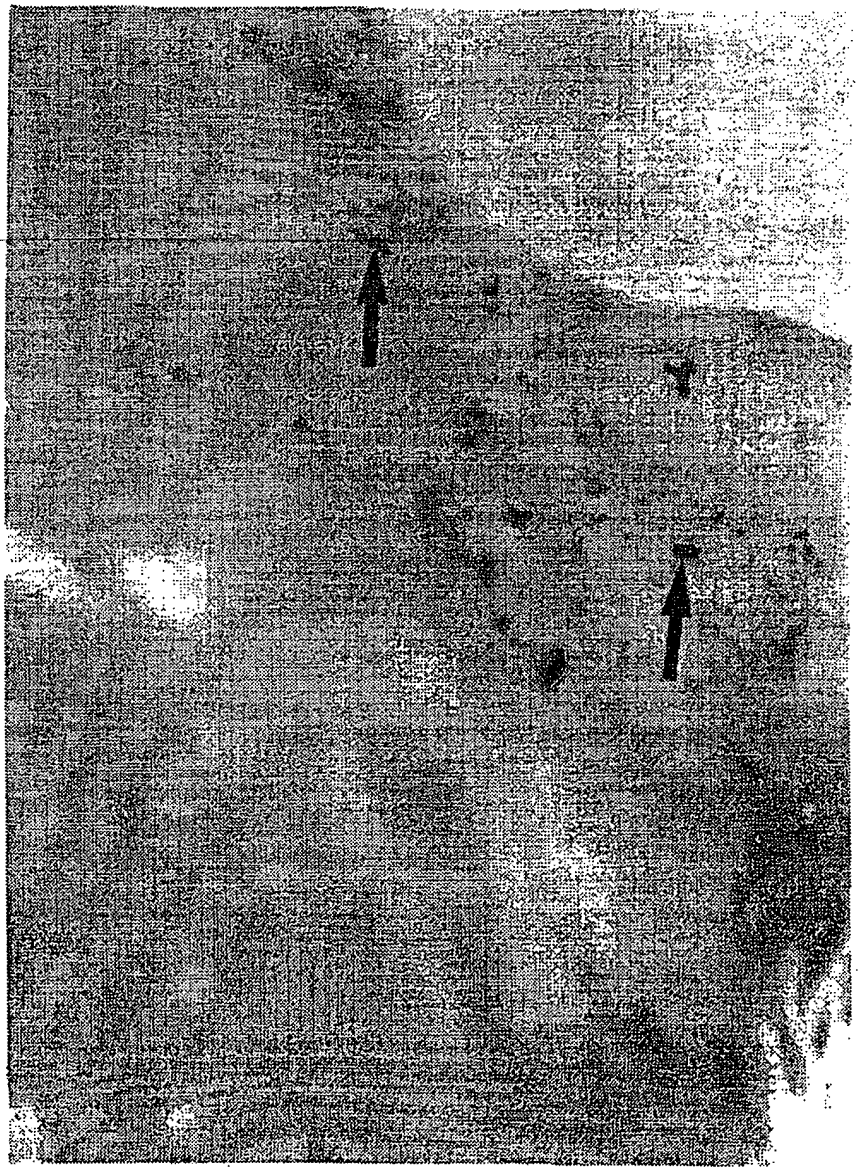


FIG.5

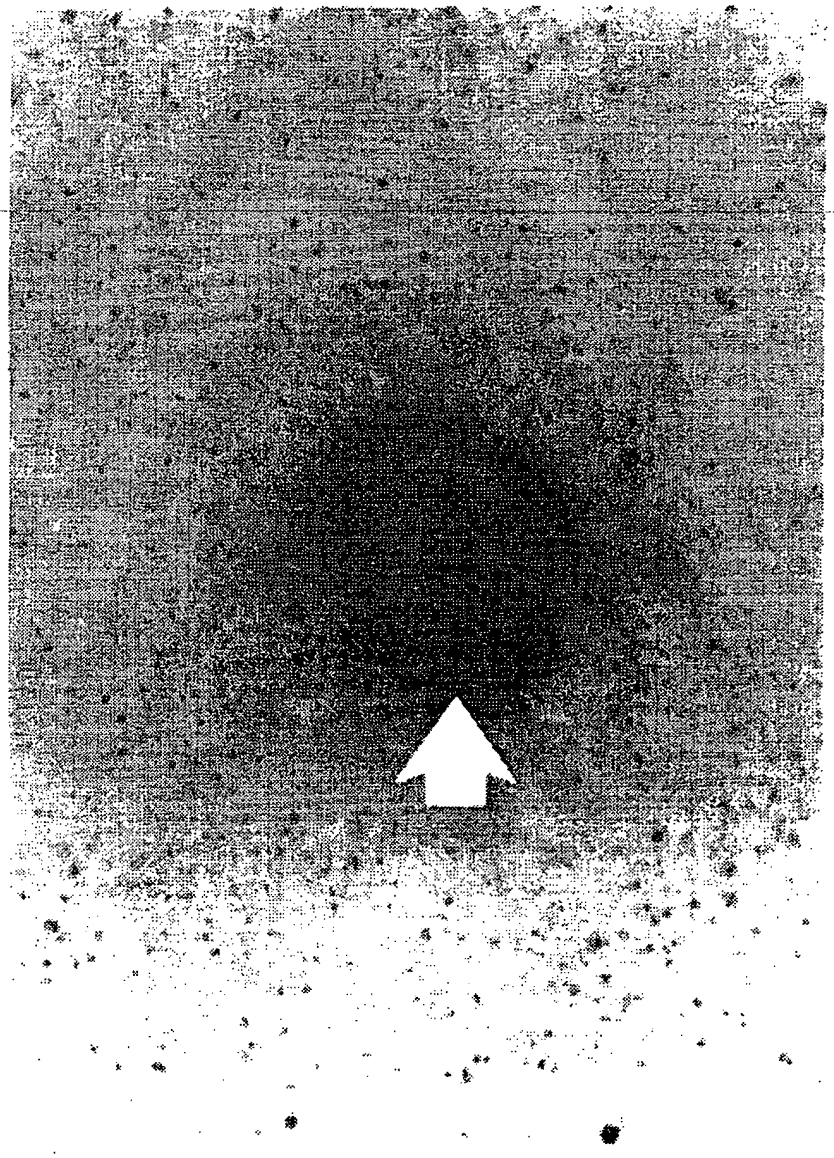
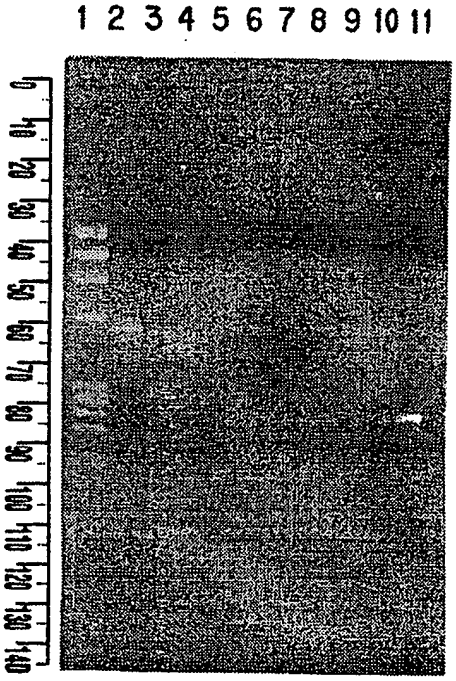


FIG.6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04239

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL : 424/88, 450, 490; 435/172.3 ; 514/44; 530/391.1, 402; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 450, 490; 435/172.3 ; 514/44; 530/391.1, 402; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/10448 (BISCHOFBERGER ET AL.) 20 September 1990, see entire document.	1-9
Y	The Journal of Biological Chemistry, Volume 263, Number 29, issued 15 October 1988, G.Y. Wu et al., "Receptor-mediated gene delivery and expression in vivo.", pages 14621-14624, see entire document.	2,6
Y	Proceedings of the National Academy of Sciences USA, Volume 88, issued April 1991, Williams et al., "Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles", pages 2726-2730, see entire document,	3,4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 JUNE 1994	Date of mailing of the international search report JUN 23 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RON SCHWADRON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04239

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 37/02, 37/22, 9/52, 31/70, 35/14, 39/00, 39/395, 48/00; CO7K 7/00, 13/00, 15/28; CO7H 17/00, 21/04

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, MEDLINE, CHEM AB, DERWENT WPI, EMBASE, search terms: author names, RNA, DNA, microparticle, coated, encapsulated, conjugate, conjugated, time release, receptor mediated, antibody, receptor, ligand